THE ROLE OF DNA SYNTHESIS IN THE DETERMINATION OF AXIAL POLARITY OF REGENERATING PLANARIANS[1]

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It has been shown that treatment of intact planarians with colcemide (deacetylmethylcolchicine) and chloramphenicol, followed by cutting the worms to remove the heads and tails, will cause the regeneration of bipolar heads in a significant number of these cut planarians (Flickinger, 1959). When applied locally to the prospective anterior ends of cut pieces of worms, these compounds could reverse the normal polarity of the worms (Flickinger, 1959; Flickinger and Coward, 1962). It was further shown that both of these compounds inhibit the extent of incorporation of $C^{14}O_2$ into a trichloroacetic acid-insoluble residue, which is primarily protein. The classical action of colchicine, or its derivatives, is to inhibit cell division, while the action of chloramphenicol in bacteria is to inhibit protein synthesis without significantly inhibiting nucleic acid synthesis (Gale and Folks, 1953; Midgley and McCarthy, 1962). An accumulation of RNA in bacteria treated with chloramphenicol has been observed (Dubin and Elkart, 1965) and such RNA has some similarities to messenger RNA (Hahn and Wolfe, 1962).

One purpose of this investigation was to find the effects of chloramphenicol upon RNA and DNA synthesis of the planarians. Does chloramphenicol stimulate or inhibit RNA synthesis? Is there an inhibition of DNA synthesis by chloramphenicol? If so, what is the per cent of inhibition of DNA synthesis after periods of exposure which cause bipolar head formation, compared to shorter times of exposure which do not affect polarity of the regenerating worms? Another aim of the present investigation was to determine the temporal course of DNA, RNA and protein synthesis in the regenerating head and tail blastemata during the first 24 hours after cutting the worms. A study of RNA and protein synthesis in regenerating planarians has been made (Coward and Flickinger, 1965), but these determinations were made at daily intervals for a 7-day period.

Materials and Methods

The planarians, *Dugesia tigrina,* maintained in aerated tap water, were starved for 7–10 days before being used in an experiment. The effect of chloramphenicol upon RNA and DNA synthesis was tested by exposing 100 worms for 12 or 24 hours to 25 μc./ml. of uridine-2-C^{14} or C^{14}-thymidine in 1 ml. of boiled tap water containing chloramphenicol succinate (1.5 mg./ml.). A similar number of control worms was cultured for similar periods in boiled tap water, containing the labeled compounds. At the conclusion of the incubation period the worms were washed five times with boiled tap water, by which time the final rinse water had a level

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of activity similar to the background. The worms were then homogenized in cold 5% trichloroacetic acid (TCA) and the pellet washed three times by centrifugation, which reduced the level of isotopic activity of the wash to that of the background. The residue was extracted twice with 1:1 ethanol-ether, twice with 1:4 ethanol-ether, and once with ether alone to remove lipids. The residues were then dried. For the C^{14}-uridine experiments RNA was hydrolyzed with 1 ml. of 0.3 N KOH at 37° C. for 20 hours. The hydrolysate was acidified with 0.3 ml. of 1 N perchloric acid to precipitate the DNA and protein and the supernatant was neutralized with 1 N KOH to precipitate the perchlorate. The concentrations of RNA were determined by the orcinol method (Dische, 1955) and the samples were plated and counted on a thin window proportional counter. The procedure for the C^{14}-thymidine incubations was similar except that both RNA and DNA were hydrolyzed with hot trichloroacetic acid (96° C.) for 40 minutes and the hydrolysate was extracted three times with ether to remove the trichloroacetic acid. The diphenylamine method (Dische, 1955) was used to determine DNA concentration before the samples were plated and counted.

For the incubations with $C^{14}O_2$ 100 cut worms were transferred to 1 ml. of recently boiled tap water in a small dish. A small amount of lactic acid was added to generate $C^{14}O_2$ from 150 μc. of $BaC^{14}O_3$ in a center well and the dish was quickly sealed to prevent the escape of $C^{14}O_2$. After the incubation, the worms were processed as before for hydrolysis of RNA with 0.3 N KOH, and DNA of the residue was hydrolyzed with hot TCA. The protein residue from the DNA hydrolysis was dissolved in formic acid, the protein concentration determined (Lowry *et al.*, 1951) and then this fraction was plated and counted.

Results

Biological experiments with chloramphenicol

In experiments designed to find the minimal concentration of chloramphenicol that would produce bipolar heads in regenerating planaria, 50 whole worms were incubated in boiled tap water containing 0.1% penicillin and streptomycin and 50 were placed in a similar solution containing chloramphenicol succinate (1.5 mg./ml.). This concentration of chloramphenicol succinate is equivalent to 1 mg./ml. chloramphenicol. Similar numbers of worms were cultured in 0.75, 0.325 and 0.167 mg./ml. of chloramphenicol succinate. After 24 hours of incubation the worms were each cut just back of the head and in front of the pharynx and the cut pieces were allowed to regenerate in boiled tap water containing 0.1% penicillin and streptomycin. Thirty per cent of the cut pieces in chloramphenicol succinate (1.5 mg./ml.) subsequently developed heads at each cut end, but no bipolar heads were observed in the pieces allowed to develop in the boiled tap water alone or in the lower concentrations of chloramphenicol. In order to learn if the polarity of the regenerating worms could be affected by exposure to chloramphenicol after the worms were cut, 60 worms were cut in a similar fashion to provide pre-pharyngeal pieces and these were then placed in chloramphenicol succinate (1.5 mg./ml.) for 24 hours. Subsequent culture of these regenerating pieces provided only two cases of bipolar head formation.

To determine the minimal period of exposure to chloramphenicol necessary to

produce bipolar heads, groups of 20 whole worms were incubated in chloramphenicol succinate (1.5 mg./ml.) for periods of 2, 8, 12, 16, 20 and 24 hours and the pre-pharyngeal pieces were obtained by cutting the worms. These were allowed to regenerate in boiled tap water containing 0.1% penicillin and streptomycin and the water was changed each day. No cases of bipolar heads occurred in worms exposed to chloramphenicol succinate (1.5 mg./ml.) for less than 20 hours. Two of the worms exposed for 20 hours and eight of those exposed for 24 hours regenerated heads at each end.

Isotopic experiments with chloramphenicol

Previous work had shown that chloramphenicol inhibits the incorporation of $C^{14}O_2$ into the trichloroacetic acid-insoluble fraction of planarians (Flickinger, 1959). To ascertain the effect of chloramphenicol upon RNA synthesis, 100 intact worms were incubated separately in 1 ml. of boiled tap water containing chloramphenicol succinate (1.5 mg./ml.) and 25 μc./ml. of uridine-2-C^{14} for 24 hours at 18° C. One hundred control worms were incubated similarly except that chloramphenicol was not present. The RNA was hydrolyzed and counted and the results show that worms in chloramphenicol incorporated less C^{14}-uridine into the RNA fraction (Table I). There was a 24.8% inhibition based on the activity/100 worms, while there was a 28.8% inhibition on the basis of specific activity.

A similar type of experiment was performed to find the effect of chloramphenicol upon DNA synthesis. One group of 100 worms was incubated in boiled tap water containing 25 μc./ml. of C^{14}-thymidine and chloramphenicol succinate (1.5 mg./ml.) for 24 hours at 18° C. while the incubation of the control group of 100 worms did not contain chloramphenicol. Another two groups of 100 worms were incubated separately for 12 hours with 25 μc./ml. of C^{14}-thymidine, and chloramphenicol succinate (1.5 mg./ml.) was present in one of those incubations. From the biological experiments it was known that a 24-hour exposure to this concentration of chloramphenicol would produce bipolar heads in regenerating worms, while the 12-hour exposure had no effect upon polarity. This experiment offered the chance to compare the effect of the biologically active and inactive doses of chloramphenicol upon the incorporation of C^{14}-thymidine into DNA. The results of this experiment are given in Table II. There was a 39.1% inhibition of C^{14}-thymidine incorporation into DNA with a 24-hour exposure to chloramphenicol, and a 10.3% inhibition following a 12-hour exposure.

TABLE I

Effect of chloramphenicol upon incorporation of C^{14}-uridine into RNA of intact planarians. Incubation of 100 worms for 24 hours at 18° C, in boiled tap water containing 25 μc./ml. of uridine-2-C^{14}, and similar incubation of another 100 worms with chloramphenicol succinate (1.5 mg./ml.) present

	Cpm / 100 worms	Cpm / mg. RNA	Per cent inhibition of specific activity
Control worms	4378	8420	
Chloramphenicol succinate (1.5 mg./ml.) worms	3303	6000	28.8

TABLE II

Effect of chloramphenicol upon incorporation of C^{14}-thymidine into DNA of intact planarians. Incubation of four groups of 100 worms for 12 and 24 hours at 18° C. in boiled tap water containing 25 μc./ml. of C^{14}-thymidine. Chloramphenicol succinate (1.5 mg./ml.) was present in one of the 12-hour and one of the 24-hour incubations

	Cpm / 100 worms	Cpm / mg. RNA	Per cent inhibition of specific activity
Control, 12 hours	447	1241	
Chloramphenicol succinate, 12 hours	368	1115	10.3
Control, 24 hours	598	1245	
Chloramphenicol succinate, 24 hours	303	758	39.1

DNA, RNA and protein synthesis of blastemata

Exposure of cut worms to chloramphenicol for 24 hours did not alter their regeneration polarity, while a similar period of exposure of whole worms to chloramphenicol, followed by cutting to obtain pre-pharyngeal pieces, produced bipolar heads in 40% of the regenerating worms. This suggested that the mechanisms acounting for establishment of normal polarity operate during the first 24 hours after cutting the worms. The rates of DNA, RNA and protein synthesis during this period were examined in the following manner. For each experiment 100 worms were cut transversely in front of the pharynx at level X (Fig. 1) and the anterior and posterior parts of these worms were allowed to regenerate for 2, 4, 6 or 24 hours in boiled tap water. $C^{14}O_2$ was generated from 150 μc. of $BaC^{14}O_3$ and the dish was sealed and incubated at 18° C. for three hours. The cut worms were then washed five times with boiled tap water, fixed in 5% TCA and cut into A_2, A_1, P_1, and P_2 parts, according to Figure 1. The head and tail blastemata (P_1 and A_1), and the areas of tissues adjacent to the head and tail blastemata (P_2 and A_2) were homogenized separately in cold 5% TCA and the centrifugal residues were washed four times by centrifugation and washing with cold 5% TCA. The DNA, RNA and protein fractions were prepared according to previously outlined methods and these fractions were plated and counted. Orcinol tests revealed the absence of RNA in the DNA fraction and the diphenylamine reaction showed there was no DNA in the RNA fraction. Examination of the results (Table III) reveals only slight differences of incorporation of $C^{14}O_2$ into DNA, RNA and protein of the four areas of the worms which had regenerated two hours without label and three hours with the label. The blastemata (P_1 and A_1) and adjacent areas (P_2 and A_2) obtained from cut worms that had regenerated 4 and 6 hours without label, plus three hours in labeled $C^{14}O_2$, showed significant differences in the levels of incorporation of label into DNA, RNA and protein. By this time the DNA, RNA and protein fractions of regenerating areas (P_1 and A_1) had higher isotopic activities than the non-regenerating areas (P_2 and A_2). After 6 hours of regeneration without $C^{14}O_2$, and three hours with $C^{14}O_2$, the head blastema (P_1) fractions (DNA, RNA and protein) incorporated more label than the tail

blastema (A_1) fractions. The non-regenerating areas near the head blastemata had higher activities than similar areas near the tail blastemata ($P_2 > A_2$). The greatest amount of $C^{14}O_2$ incorporation into DNA occurred at 4 hours for the tail blastemata (A_1) and the adjacent area (A_2), while the incorporation of label into both DNA and RNA of all the other fractions was greatest at 6 hours. In comparing the levels of isotope incorporation of the worms allowed to regenerate two hours in unlabeled medium and three hours in $C^{14}O_2$ with those in which the worms regenerated four or six hours in unlabeled medium and three hours in $C^{14}O_2$, significant increases of incorporation of labeled precursor into DNA and RNA were observed in the four- and six-hour experiments. Furthermore, there is a marked stimulation of DNA synthesis in the blastemata of worms that regenerated four hours in unlabeled medium, but no further significant stimulation of labeled precursor into DNA after six hours of regeneration in unlabeled medium. The extent of stimulation of RNA synthesis, particularly in the anterior pieces (P_1 and P_2), was greater between four and six hours of regeneration in unlabeled medium, as compared to the difference between two and four hours of regeneration in the unlabeled medium. The specific activities of the protein fraction were maximal after 24 hours of regeneration in tap water and three hours with the label. The differences in isotopic activities of the protein fraction between the head and tail blastemata

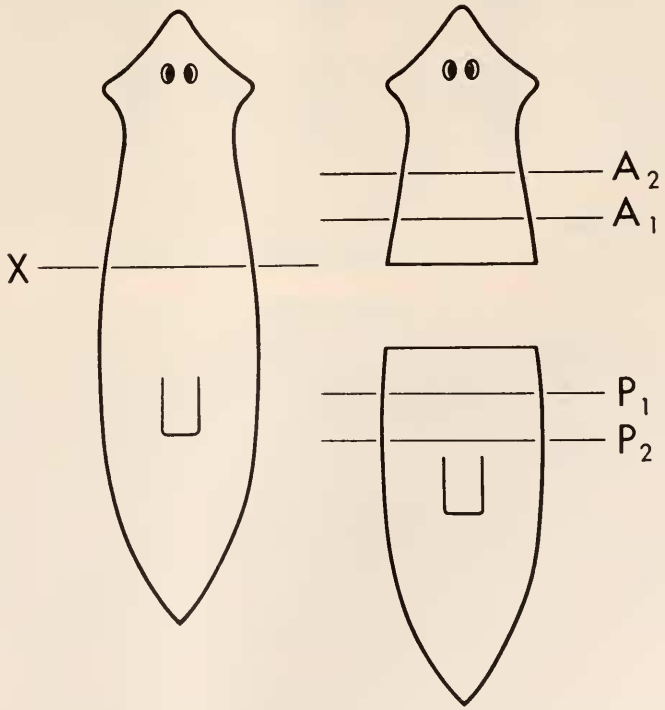

FIGURE 1. For each experiment 100 worms were cut at level x and allowed to regenerate for 2, 4, 6 and 24 hours. The blastemata (A_1 and P_1) and adjacent areas (A_2 and P_2) were cut and then incubated separately for three hours in 1 ml. of boiled tap water containing $C^{14}O_2$ generated from 150 μc. of $BaC^{14}O_3$.

Table III

Incorporation of $C^{14}O_2$ into DNA, RNA and protein of head and tail blastemata and adjacent regions during the first 24 hours of regeneration. For each experiment 100 worms were cut anterior to the pharynx (Fig. 1) and the pieces allowed to regenerate for 2, 4, 6 or 24 hours at 18°C. The pieces (A_1, A_2, P_1, P_2) were then cut according to Fig.1 and the pieces of each kind were incubated for 3 hours at 18°C. in 1 ml. of boiled tap water with $C^{14}O_2$ generated from 150 μc. of $BaC^{14}O_3$

Tissue	Cpm/mg.			
	Time			
	2 hrs.	4 hrs.	6 hrs.	24 hrs.
DNA				
Tail blastema A_1	14370	35140	33900	18700
A_2	15140	27290	25600	16800
Head blastema P_1	14340	39780	40800	22760
P_2	13500	25680	29400	13660
RNA				
Tail blastema A_1	17860	25350	32430	30913
A_2	15230	21300	26955	22220
Head blastema P_1	18490	21390	43250	35880
P_2	17640	17160	28980	28500
Protein				
Tail blastema A_1	1370	1520	1285	1820
A_2	1250	1088	1080	1340
Head blastema P_1	1445	1863	1760	2840
P_2	1130	1187	1440	2600

($P_1 > A_1$), the adjacent areas ($P_2 > A_2$), and the regenerating and non-regenerating areas ($P_1 > P_2$; $A_1 > A_2$), were also maximal at 24 hours.

Discussion

The results of this investigation show that dosages of chloramphenicol succinate (1.5 mg./ml. for 24 hours) that can induce the formation of bipolar heads in regenerating planarians inhibit RNA and DNA synthesis (Tables I and II). The inhibition of incorporation of C^{14}-uridine into RNA (Table I) argues against the idea that the morphogenetic activity of chloramphenicol is due to an accumulation of RNA, as can occur in bacteria (Dubin and Elkart, 1965). Incubation of the worms with labeled thymidine and chloramphenicol succinate (1.5 mg./ml.) for 24 hours revealed a severe inhibition (39.1%) of DNA synthesis (Table II). Exposure of whole worms to this same concentration of chloramphenicol for 12 hours does not alter the normal polarity during subsequent regeneration and this length of exposure to chloramphenicol resulted in a 10.3% inhibition of DNA synthesis (Table II). The data from these experiments show that a marked inhibition of DNA synthesis by chloramphenicol is essential in order to alter the polarity of the regenerating worms. Previous work has shown that exposure of intact worms to the mitotic poison, colcemide, can affect the polarity of these worms during their

regeneration (Kanatani, 1958; Flickinger, 1959) and emphasizes that cell division is a critical factor for maintenance of normal polarity.

Exposure of cut worms to chloramphenicol succinate (1.5 mg./ml.) for 24 hours did not alter their normal polarity. This agrees with the results of Kanatani (1958) who found that colcemide did not alter polarity when applied to cut worms. This suggests that the critical events that maintain polarity in cut pieces of planarians are established sometime during the first 24 hours after the worms are sectioned. Investigation of $C^{14}O_2$ incorporation of head and tail blastemata (P_1 and A_1), and adjoining areas (P_2 and A_2), revealed that DNA, RNA and protein synthesis are stimulated 7–9 hours after cutting the worms. Furthermore, the head blastema (P_1) has a significantly higher incorporation of $C^{14}O_2$ into these fractions than the tail blastema (A_1) by this time. In reference to the 2–5-hour levels of incorporation the greatest stimulation of DNA synthesis occurred 4–7 hours after cutting and the maximal increase of RNA synthesis from 6–9 hours after cutting, while the maximal incorporation of $C^{14}O_2$ into the protein fractions occurred 24–27 hours after cutting. It appears that the rate of protein synthesis is maximal at the time of actual differentiation while the rates of DNA and RNA synthesis increase during the period of the determination of axial polarity. It does seem that the time of greater stimulation of DNA synthesis precedes the time of maximal stimulation of RNA synthesis, using the two-hour period of regeneration in unlabeled medium as the reference.

It has been found that the action of chloramphenicol in producing bipolar heads is correlated with a severe inhibition of DNA synthesis, and that one of the first metabolic activities to show maximal stimulation during regeneration is DNA synthesis. This suggests that cell division plays an important role in the establishment of axial polarity in planarians. However, cytological data relating to mitotic frequency are necessary to confirm this suggestion.

Summary

1. Levels of chloramphenicol which can produce bipolar heads in regenerating planarians were found to inhibit severely the synthesis of both DNA and RNA. A shorter period of exposure to chloramphenicol, which did not affect the polarity of the regenerating worms, produced only a slight inhibition of DNA synthesis.

2. Incubation of blastemata and adjacent areas of regenerating planarians with labeled CO_2 for periods of 2, 4, 6 and 24 hours revealed a maximal stimulation of DNA at 4–7 hours and of RNA at 6–9 hours after the worms were cut. It is at this time that the blastemata destined to form heads attain a higher level of DNA and RNA synthesis than the blastemata which will form tails. The maximal stimulation of protein synthesis occurred 24–27 hours after cutting the worms.

3. The necessity of obtaining a severe inhibition of DNA synthesis with chloramphenicol in order to produce bipolar heads in regenerating worms, as well as the sequential nature of DNA, RNA and protein synthesis in the regeneration blastemata, suggests that the stimulation of DNA synthesis is involved in the establishment of polarity of regenerating worms. These patterns of DNA synthesis may reflect the incidence of cell division during this period.

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